

**Temporary feeding shocks increase the productivity in a continuous  
biohydrogen producing reactor**

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## Abstract

Continuous hydrogen production stability and robustness by dark fermentation were comprehensively studied at laboratory scale. Continuous bioreactors were operated at two different hydraulic retention times (HRT) of 6 and 10 hours. The reactors were subjected to feeding shocks given by decreases in the HRT, and therefore the organic loading increase, during 6 and 24 hours. Results indicated that the  $H_2$  productivity was significantly improved by the temporary organic shock loads, increasing the hydrogen production rate up to 40%, compared to the rate obtained at the steady-state condition. Besides, it was observed that after the shock load, the stability of the reactor (measured as the hydrogen production rate) was recovered attaining the values observed before the feeding shocks. The bioreactor operated at shorter HRT (6 h) showed better  $H_2$  productivity ( $17.3 \pm 1.1$  L  $H_2$ /L-d) in comparison to the other one operated at 10 h HRT ( $12.4 \pm 1.6$  L  $H_2$ /L-d).

**Keywords:** biohydrogen, CSTR, dark fermentation, feeding shock, HRT perturbations, statistical analysis

## 1. Introduction

Dark fermentation has been extensively investigated in the past decades and from a practical point of view, it became one of the most feasible methods for biohydrogen production (Kumar et al. 2015). As a result of the significant progress on these methods, the need of producing biohydrogen in continuous operation was emphasized since it is preferred for future larger-scale implementations (Wang and Wang 2009). Nevertheless, to accomplish sustainable biotechnological hydrogen formation in continuous systems, several factors and process variables have to be considered (Bakonyi et al. 2014). For instance, the selection, enrichment and adaption of inoculum (Hernández-Mendoza and Buitrón 2014; Hernández-Mendoza et al. 2014; Kumar et al. 2016), the reactor design (Bakonyi et al. 2014) and the operating conditions e.g. hydraulic retention time (HRT) (Buitrón et al. 2014; Bakonyi et al. 2015; Sivagurunathan et al. 2016).

Buitrón and Carvajal (2010) reported that short hydraulic retention times increased the biohydrogen production. Specifically, Thanwised et al. (2012) achieved a rise in hydrogen productivity (164 vs. 883 mL H<sub>2</sub>/L-d) by switching the HRT from 24 h to 6 h in an anaerobic baffled reactor. Also, Ramírez-Morales et al. (2015) accomplished maximum mean hydrogen productivity of 23.4 L H<sub>2</sub>/L-d at 6 h of HRT in a continuous stirred tank reactor (CSTR). However, Park et al. (2015) communicated that short hydraulic retention times could be seen as a threat given that may cause the loss of precious H<sub>2</sub>-forming biological activity. Shen et al. (2009)

assessed the effect of the Organic Loading Rate (OLR) on the biohydrogen production using two reactor configurations (CSTR and a membrane bioreactor), achieving a maximum productivity of 4.25 LH<sub>2</sub>/L-d at 30 g chemical oxygen demand (COD)/L-d in the CSTR, whilst the productivity was of 4.48 LH<sub>2</sub>/L-d at 22 g COD/L-d in the membrane bioreactor. Hafez et al. (2010) reported maximum H<sub>2</sub> productivity of 35.6 L H<sub>2</sub>/L-d at 103 g COD/L-d in a CSTR coupled to a clarifier. Later, Zhang (2014) accomplished maximum hydrogen productivity (22.8 L H<sub>2</sub>/L-d) at approx. 85 g COD/L-d in a CSTR. Besides, Ramírez-Morales et al. (2015) evaluated the effect of OLR on the hydrogen production in a CSTR using glucose as substrate, and documented 25.4 L H<sub>2</sub>/L-d productivity at 100 g COD/L-d.

Nevertheless, the long-term stability of the fermenter is considerably dependent on the process conditions, mainly the HRT and the OLR. The possible vulnerability of continuous hydrogen fermenters was enlightened by Baghchehsaraee et al. (2011), revealed after careful experimentation that perturbations in the process associated with feed interruption could strongly affect the process stability and revival of bacteria. Hence, when extreme disturbances occur, the biosystem – depending on its robustness – may fail. Although the probability of operational disturbances under laboratory environment is expectably low because of the well-controlled input and process variables i.e. constant feed composition and feed flow rate, process disturbances could be notable when stepping out to a real and industrial case (Fig. 1). For instance, Krupp and Widmann (2009) pointed out that reliable feeding of the bioreactor could be challenging due to

difficulties with (i) pumping, (ii) mixing or (iii) the supply of a consistent input stream. However, such aspects are scantily addressed in the literature.

This work evaluates the continuous biohydrogen production by dark fermentation with a primary focus on the impact of organic shock loads associated with shifts in the HRT. Besides, the duration of these temporary feeding shocks as another variable of operational disturbance was assessed as well. Data and experiences about the influence of temporary process shocks could have a useful contribution to the design of future, scaled-up continuous processes for biohydrogen production.

## 2. Materials and Methods

### 2.1 Inoculum, culture medium and start-up

According to literature reports, the employment of diverse, mixed bacterial populations in completely stirred tank reactors is an attractive and widely-applied strategy to start-up and establish continuous hydrogen production (Jung et al. 2011; Show et al. 2011). In this study, granular anaerobic sludge from a UASB reactor for wastewater treatment was used as inoculum after thermal pretreatment (Buitrón and Carvajal, 2010) for an H<sub>2</sub>-producing CSTR operated at HRT=12 h under steady-state at the lab. This inoculum had been characterized by pyrosequencing in previous work (Bakonyi et al., 2017), finding the dominance of hydrogen-producing

microorganisms such as *Clostridium pasteurianum* and *Pectinatus frisingensis* in the microbial community.

Adopting the sludge from the reactor used in the work of Bakonyi et al. (2017), two CSTRs were started-up in this work, differing in process HRT and corresponding OLR. In the two separate reactors (referred as Reactor 1 and 2 onward), HRT of 6 h and 10 h were adjusted, respectively. Corresponding OLRs were 85.6 g COD/L-d and 51.4 g COD/L-d, employing glucose as a substrate in the culture medium at a constant concentration of 20 g/L (1.07 g COD/g glucose). Besides glucose, as carbon source, for every liter of feed solution, the following amounts of mineral salts – modified from Ramírez-Morales et al. (2015) – were supplied: K<sub>2</sub>HPO<sub>4</sub>, 50 mg; NH<sub>4</sub>Cl, 104 mg; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.4 mg; MgCl<sub>2</sub>·6H<sub>2</sub>O, 20 mg; FeSO<sub>4</sub>·7H<sub>2</sub>O, 20 mg; CoCl<sub>2</sub>·6H<sub>2</sub>O, 2 mg; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 2 mg; H<sub>3</sub>BO<sub>4</sub>, 2 mg; NiCl<sub>2</sub>·6H<sub>2</sub>O, 2 mg; ZnCl<sub>2</sub>, 2 mg. The media was prepared using tap water and refrigerated at 4 °C to minimize fluctuations in its composition.

Both Reactors 1 and 2, had 1.25 L/0.9 L total/working volumes. First, the CSTRs operated in batch mode for 24 h with 10 g glucose/L at initial volatile suspended solid (VSS) concentration of 4 g/L. Afterward, the reactors were switched to continuous mode and the glucose concentration was increased to 20 g/L in the medium and maintained constant onward, as mentioned above. Steady-state was considered once stable H<sub>2</sub> production (± 10-15 % daily variation) could be reached at least for 2 consecutive days (Lin et al., 2008).

The reactors were equipped with an EZ-Bioreactor Control device (Applikon Biotechnology, Schiedam, The Netherlands) and operated at 35 °C. 100 rpm stirring rate by an upper shaft agitator with 2 equally spaced Rushton turbines was ensured. The pH was maintained at 5.5 by 3M NaOH and 3M HCl solutions. A conductivity-based on/off level sensor was built-in to precisely control the liquid level. The scheme of the experimental apparatus was based on earlier work (Ramírez-Morales et al. 2015) and is shown in **Fig. 2**. Initially, the bioreactors were purged with >99.9 % N<sub>2</sub> to establish fully anaerobic conditions.

## 2.2. Selection of conditions for perturbation tests (OLR, HRT)

The following experimental plan was executed to test reactor stability and robustness over time as a response to feeding perturbations. In both Reactor 1 and 2, shocking HRTs of 5 and 3 hours were applied, resulting in OLRs of 102.7 and 171.2 g COD/L-d, respectively. The durations were varied either as 6 or 24 hours. These operating conditions were chosen according to the presented literature and previous results.

Concerning the OLR selection, **Fig. 3** shows the hydrogen productivity at several OLR values tested by various authors. Accordingly, an OLR between 90 and 120 g COD/L-d seems to foster the biohydrogen production. Nonetheless, above this range (>120 g COD/L-d), a disadvantageous effect can occur, depressing the H<sub>2</sub> productivity. Hence, one perturbing OLR value (102.7 g COD/L-d) that falls to the



90-120 g COD/L-d range has been selected, as well as one value that is out of such range and likely disadvantageous (171.2 g COD/L-d).

Regarding the HRT and glucose input concentration selection, Ramírez-Morales et al. (2015) reported maximum hydrogen productivity of 25.4 L H<sub>2</sub>/L-d at HRT=4 h and under an optimum value of OLR (100 g COD/L-d) given by a fixed substrate concentration (16.7 g glucose/L). Moreover, Villa-Leyva (2015) found that at 25 g glucose/L and 106.7 g COD/L-d (HRT=6 h), glucose consumption by the biological system decreased from 97% to 81% due to substrate overload, using a CSTR and the same type of sludge and inoculum pretreatment than in this research.

Furthermore, Valdez-Vazquez and Poggi-Varaldo (2009) documented the specific growth rate for both hydrogen-producing bacteria (0.083 h<sup>-1</sup>) and methanogenic archaea (0.0167 h<sup>-1</sup>), which diminish the hydrogen production. In this sense, it was convenient to operate reactors at high dilution rates and hence low HRT (around 12 h or even lower) to promote the washout of methanogenic archaea and prevent their growth. These results were the basis for setting the experimental conditions of this work (regarding shocking HRT and OLR) to even suboptimal values to assess the H<sub>2</sub>-producing capacity of either bioreactors or the biological system.

## 2.3 Analytical methods

The composition of biogas samples (hydrogen, carbon dioxide and methane contents) was determined by gas chromatography (SRI Model 8610c) using thermal conductivity detector. The analysis of volatile fatty acids – acetic acid (HAc), butyric acid (HBu) – was performed on a gas chromatograph (Agilent technologies model 7890B) connected to a flame ionization detector. Both methodologies were followed as described by [Buitrón and Carvajal \(2010\)](#). The volume of biogas produced was measured by a MilliGascounter (Ritter, Germany) connected to the COM1 serial port of a desk computer (PC). Glucose consumption was followed by the phenol/sulfuric acid method ([DuBois et al. 1956](#)).

## 2.4 Calculations

H<sub>2</sub> productivity was calculated according to Eq. 1:

$$P_{H_2} = V_H / (V_R t) \quad (1)$$

where P<sub>H<sub>2</sub></sub> is the H<sub>2</sub> productivity (L H<sub>2</sub>/L-d), V<sub>H</sub> is the volume of H<sub>2</sub> produced (L), V<sub>R</sub> is the reactor working volume (L) and t is the operational time or sampling time for each H<sub>2</sub> volume measurement. Volumes of H<sub>2</sub> are referred to Standard

Temperature and Pressure (STP) conditions (273.15 K and 100 kPa, respectively).

On the other hand, H<sub>2</sub> yield was calculated according to Eq. 2:

$$Y_{H_2} = n_H/n_G \quad (2)$$

where n<sub>H</sub> and n<sub>G</sub> are the amounts of hydrogen produced (mol) and glucose consumed (mol), respectively.

## 2.5 Statistical evaluation

A statistical approach was applied to assess the impact of process perturbations caused by feeding shocks (both the shocking HRT and its duration) on the hydrogen production rate (HPR) regarding the statistically significant difference. This approach consisted of performing a two-way analysis of variance (ANOVA) to the whole H<sub>2</sub> productivity data for each reactor separately with the aim of finding any statistical difference between either HRT or the feeding shock duration on the HPR.

In this sense, Bartlett test was also applied to check the homogeneity of variances in each group of data. This test consists of statistical analysis about a variance to find out if variances among the experimental treatments are equal or not. The Bartlett statistic allows accepting the hypothesis of equal variances among the

different levels of a variable when it is below or equal to  $X^2_{\alpha;\alpha-1}$ . The statistical analysis was conducted using R software.

### 3. Results and Discussion

#### 3.1 Performance of continuous hydrogen producing bioreactors under non-disturbed and shocking conditions

In Reactor 1 (operated at HRT=6 h, OLR=85.6 g COD/L-d), steady-state was reached after 2 days operation, achieving average hydrogen productivity of 17.3 L H<sub>2</sub>/L-d. Then, the reactor was subjected to four organic shock loads (two at HRT=5 h, OLR=102.7 g COD/L-d during 6 and 24 hours respectively, and two at HRT=3 h, OLR=171.2 g COD/L-d with the same duration), as shown in **Fig. 4**. It is important to note that no substantial increase in the hydrogen productivity could be observed after the four temporary feeding shocks, as illustrated in **Fig. 4**. Also, this set of experiments with Reactor 1 evidenced the robustness of both the biological system and reactor to withstand HRTs even as low as 3 hours for the tested period.

Considering those preliminary results, Reactor 2 (operated at HRT=10h, OLR=51.4 g COD/L-d) was perturbed with 5 and 3 hours of shocking HRT to cause higher (more intense) organic loading increases (102.7 and 171.2 g COD/L-d respectively) and evaluate the reactor robustness in such case. Once again, two different durations of the shocking HRT were assessed (6 and 24 hours). In this context, **Fig. 5** shows the associated results, where it can be observed that mean H<sub>2</sub>

productivity of 12.4 L H<sub>2</sub>/L-d was achieved during the steady state (non-disturbed operation), given by the values represented by blue dots in **Fig. 5**, which was much lower than in Reactor 1 (17.3 L H<sub>2</sub>/L-d).

Besides, it **was observed** from the outcomes that an increase in the organic loading **rate leads** the biological system towards the **enhancement of the** hydrogen production. It is noteworthy that regardless of which reactor is considered, the H<sub>2</sub> production performance was successfully recovered after each HRT/OLR perturbation, meaning that similar steady-state HPRs could be noted before and after each temporary introduced shock. The statistical analysis supported such behavior.

In this sense, two-way ANOVA was performed on the hydrogen productivity data of each reactor (Table 1), whose results revealed that both HRT (p-value=8x10<sup>-4</sup>) and shocking duration (p-value=1.2x10<sup>-3</sup>) had a significant statistical impact on the H<sub>2</sub> productivity in Reactor 2 operated at HRT=10 h. That **evidence the significant effect of the feeding shocks on the HPR in a scenario where the shocking HRT substantially different from the operating HRT.**

Concerning Reactor 1 and examining **Fig. 4**, it seems that neither the shocking HRT nor its duration had a significant effect on the HPR. The ANOVA confirms the results (Table 1, p-values of 0.967 and 0.327 respectively). Nevertheless, the interaction between both variables did have a statistically significant effect on the H<sub>2</sub> productivity (p-value=0.016) in Reactor 1, operated at HRT= 6 h, which could not be simply observed from **Fig. 4**.

Also, Bartlett test was executed to each reactor, which confirmed the homogeneity of variances in all levels of the process variables (HRT and duration) for both Reactors 1 and 2, given by p-values of 0.072 and 0.761 respectively (Table 1).

In conclusion, statistical results evidenced the increase in hydrogen production under shocking HRT, mainly in Reactor 2 (HRT=10 h) due to the higher difference between organic loads in this reactor in comparison to Reactor 1 operated at HRT=6 h. Nevertheless, in the light of mean HPRs in Reactors 1 and 2, 40% improvement was achieved in Reactor 1, which coincides with the common literature finding that shorter process HRT/higher OLR may result beneficial regarding hydrogen production. In particular, as it was already demonstrated, shortening the HRT normally in the range of 12-6 h (until a threshold level where wash out of useful bacteria could potentially occur) can enhance the H<sub>2</sub>-formation (Hawkes et al., 2002; Tapia-Venegas et al., 2013).

Furthermore, it is deduced that both systems are robust enough to endure the process shocks applied in an equally promising way. That is in agreement with the relevant literature, considering the similar robustness of a hydrogen-producing fermenter. Park et al. (2015) demonstrated that despite the various harsh disturbances subjected to the reactors (shock loading, acidification, starvation and alkalization) caused temporary changes, the original performance and therefore, the steady state could always be restored.

According to the literature (**Fig. 3**), an OLR exceeding 120 g COD/L-d may cause a process inhibition. Nevertheless, operating results presented in this research using an OLRs of 171.2 g COD/L-d, (**Figs. 4 and 5**) demonstrated that the reactors could be operated within this range (for a period up to 24 hours) without significant washout, drop in the H<sub>2</sub> productivity and deterioration of reactor stability. Thus the long-term reactor performance is not affected.

### **3.2 Correlation of H<sub>2</sub> production efficiency with VFA patterns for the 10h-HRT reactor**

By correlating the results of volatile fatty acids (acetic and butyric acids) analysis with H<sub>2</sub> production performance – for example as given in **Fig. 6A** for Reactor 2, operated at 10 h of HRT – it is implied that the hydrogen evolution (expressed as HPR) under non-disturbed conditions (steady-state under HRT=10 h) was dependent on the butyric to the acetic acid ratio (HBu/HAc). In other words, although only slight fluctuations of H<sub>2</sub> productivity were noted throughout the non-disturbed state as mentioned before, these fluctuations were always accompanied by some changes in the distribution of soluble metabolic products (HAc, HBu). Higher HBu/HAc ratios reflected those changes along the course of the process, which also promoted better gas formation efficiency (**Fig. 6A**). Such behavior agrees with the results of other literature studies communicating enhanced H<sub>2</sub> generation at higher HBu/HAc ([Chen et al. 2002](#); [Kim et al. 2006](#)), which has been a routine parameter to

judge the effectiveness of the system (Arooj et al. 2008). Moreover, it was shown that butyrate-dominant metabolism in hydrogen-producing anaerobic cultures utilizing glucose is thermodynamically favored (Lee et al. 2008).

Additionally, H<sub>2</sub> productivity had a direct relationship with hydrogen yield (Fig. 6B), which could reach values as high as 60-70 % of the practical upper-bound (4 mol H<sub>2</sub>/mol glucose) (Hallenbeck, 2009). Moreover, Fig. 6C relates the hydrogen yield against the H<sub>2</sub>Bu/H<sub>2</sub>Ac ratio revealing that in general high H<sub>2</sub>Bu/H<sub>2</sub>Ac ratios foster high H<sub>2</sub> yields. The efficiencies found regarding H<sub>2</sub> yield have been typically published for hydrogen-producing biosystems by dark fermentation with mixed microbial consortia (Lee et al. 2010).

Finally, it could be noticed that the biogas compositions – irrespective of the HRT – had no significant variations (60-63 vol.% H<sub>2</sub> and 37-40 vol.% CO<sub>2</sub>) and without methane detection. This stability in the gas composition is advantageous for biohydrogen upgrading purposes using membrane technology (Bakonyi et al. 2015, 2016). Such improving method has been shown as a promising downstream technology for the purification and concentration of H<sub>2</sub> (Bakonyi et al. 2013; Ramírez-Morales et al. 2013; Shen et al. 2009) before feeding it to efficient fuel cells for power generation.



#### 4. Conclusions

The robustness of continuous biohydrogen fermenters to process disturbances such as organic shock loads, with the concomitant HRT shocks, and their related durations was examined. It turned out that CSTR reactors fairly withstand the feeding shocks. The results indicated that the H<sub>2</sub> productivity was statistically significant (ANOVA) improved by the temporary organic shock loads, increasing the HPR up to 40%. After the applied shocks the initial average H<sub>2</sub> productivity is restored, irrespective of the shocks applied (3 and 5 hours of HRT, corresponding to 171.2 and 102.7 g COD/L-d of OLR respectively, lasting either 6 or 24 hours). The outcomes presented in this research indicate that hydrogen-producing reactors can be robust enough to withstand process shocks. Those results are useful to develop a hydrogen productivity optimization strategy.

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## Figure captions

**Fig. 1** Potential difference between laboratory and real-case fermentation conditions

**Fig. 2** Experimental continuous hydrogen production system used by Ramírez-Morales et al. (2015)

**Fig. 3** Comparison of literature studies regarding the effect of OLR on H<sub>2</sub> productivity.

**Fig. 4** Hydrogen productivity and Organic Loading Rate in Reactor 1 operated at HRT=6h subjected to two feeding shocks of 102.7 g COD/L-d (HRT=5h) and two shocks of 171.2 g COD/L-d (HRT=3h)

**Fig. 5** Hydrogen productivity and Organic Loading Rate in Reactor 2 operated at HRT=10h subjected to two feeding shocks of 102.7 g COD/L-d (HRT=5h) and two shocks of 171.2 g COD/L-d (HRT=3h)

**Fig. 6** (A) The dependency of H<sub>2</sub> production performance on butyric to acetic acid ratio (HBu/Hac), (B) The correlation of H<sub>2</sub> productivity with H<sub>2</sub> yield and (C) The relationship between hydrogen yield and HBu/Hac ratio under non-disturbed conditions in a CSTR operated with process HRT of 10 h.

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479 **Table legend**

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481 **Table 1.** Bartlett test and two-way ANOVA results on the significance of HRT and  
482 feeding shocks duration on the bioreactor performance

Figure 1

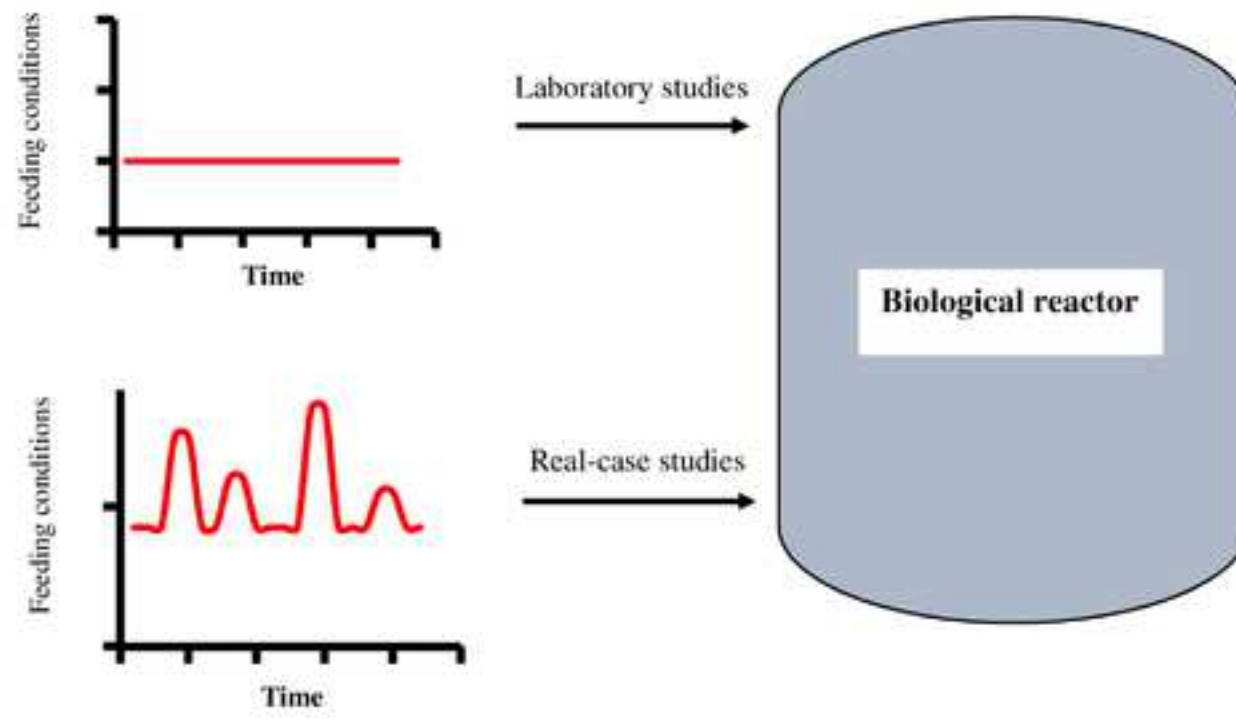


Figure 2

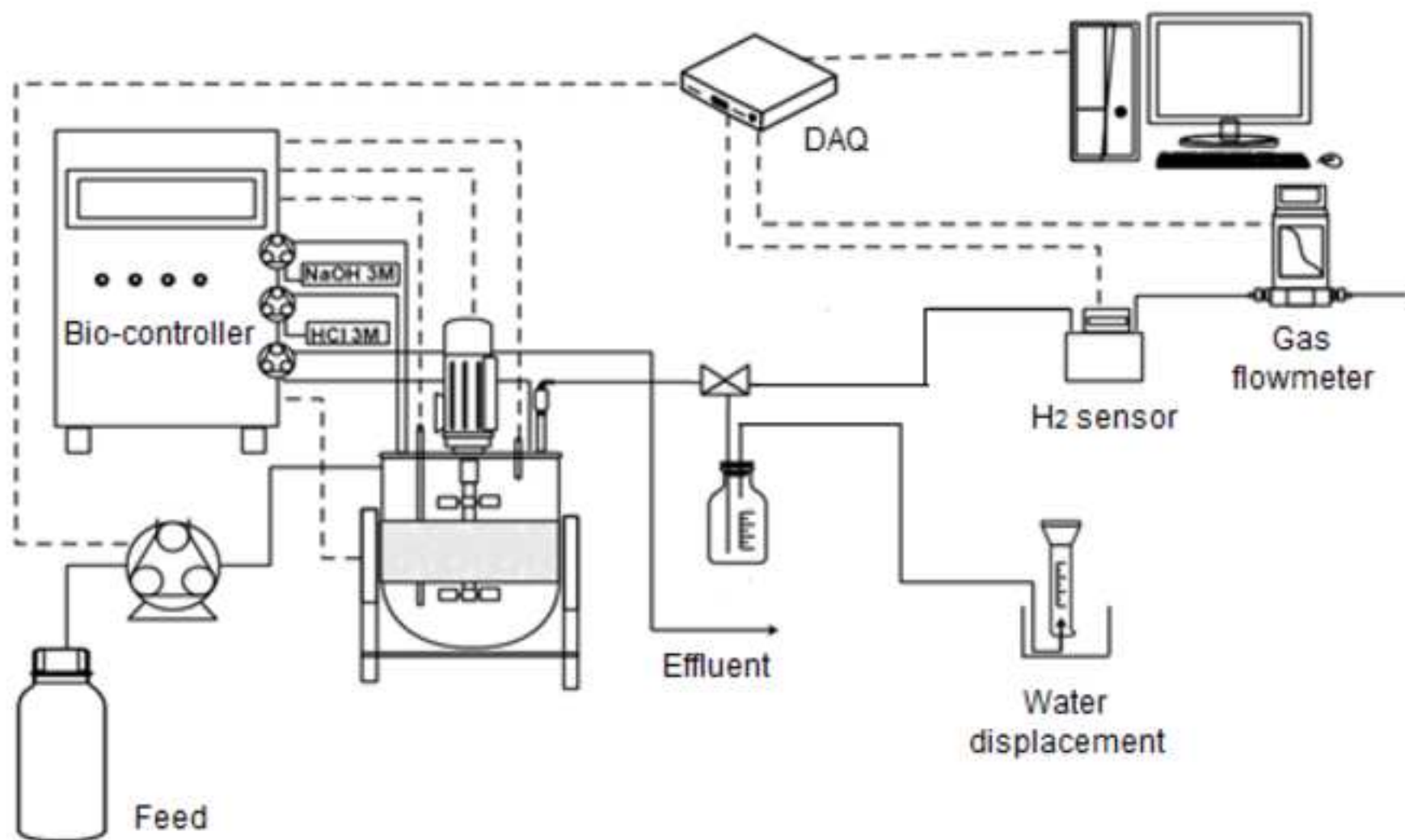


Figure 3

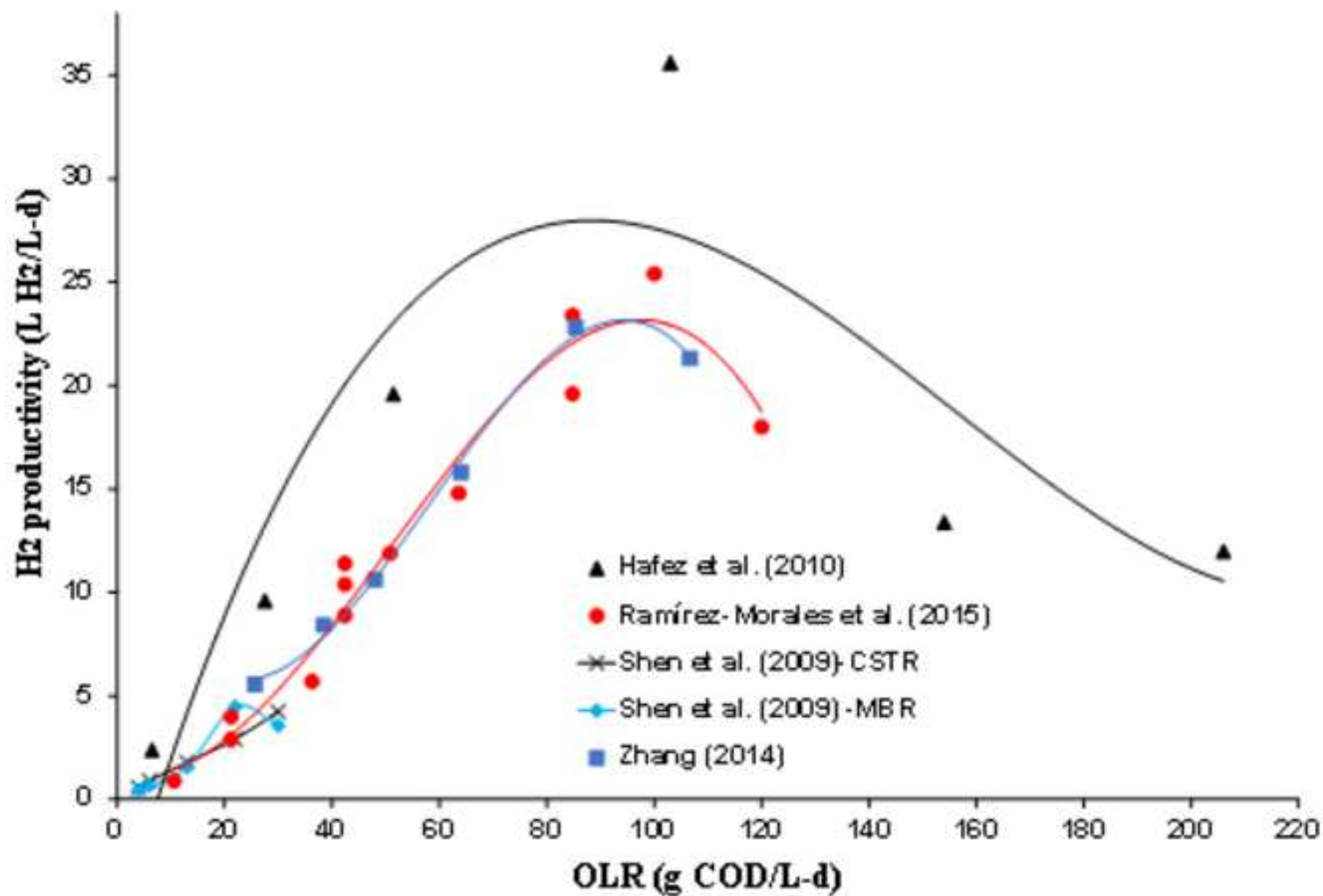


Figure 4

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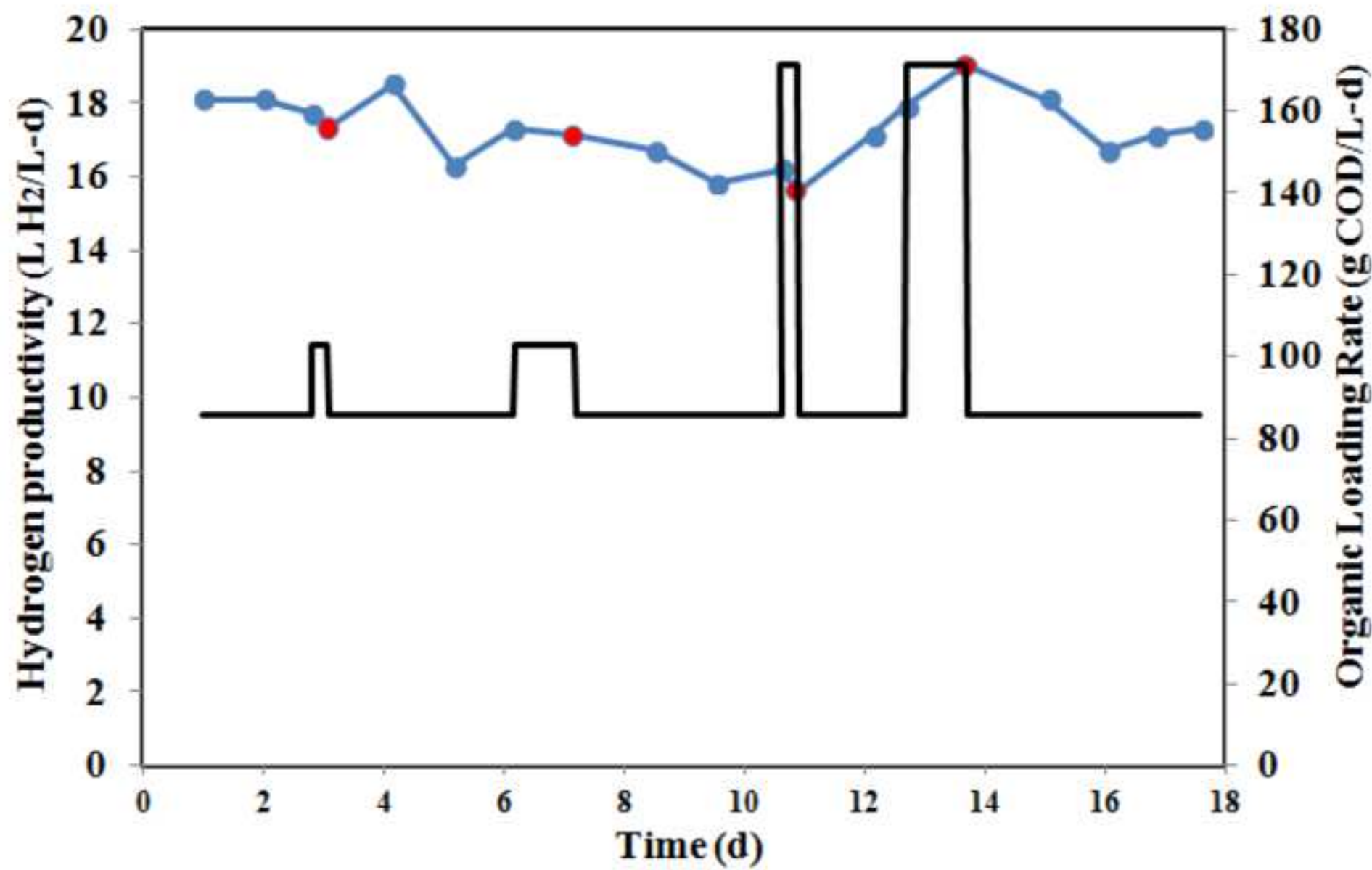


Figure 5

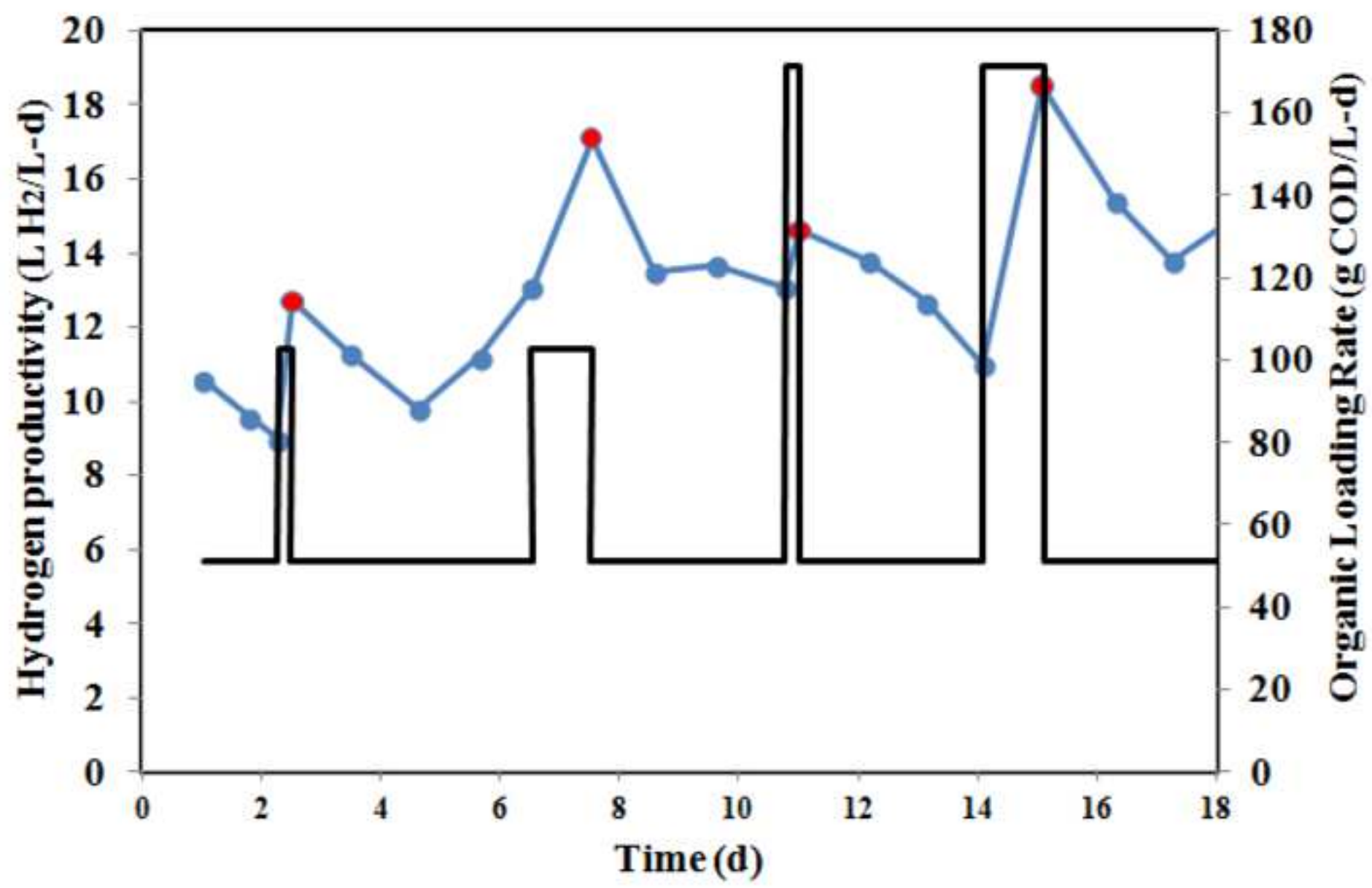


Figure 6

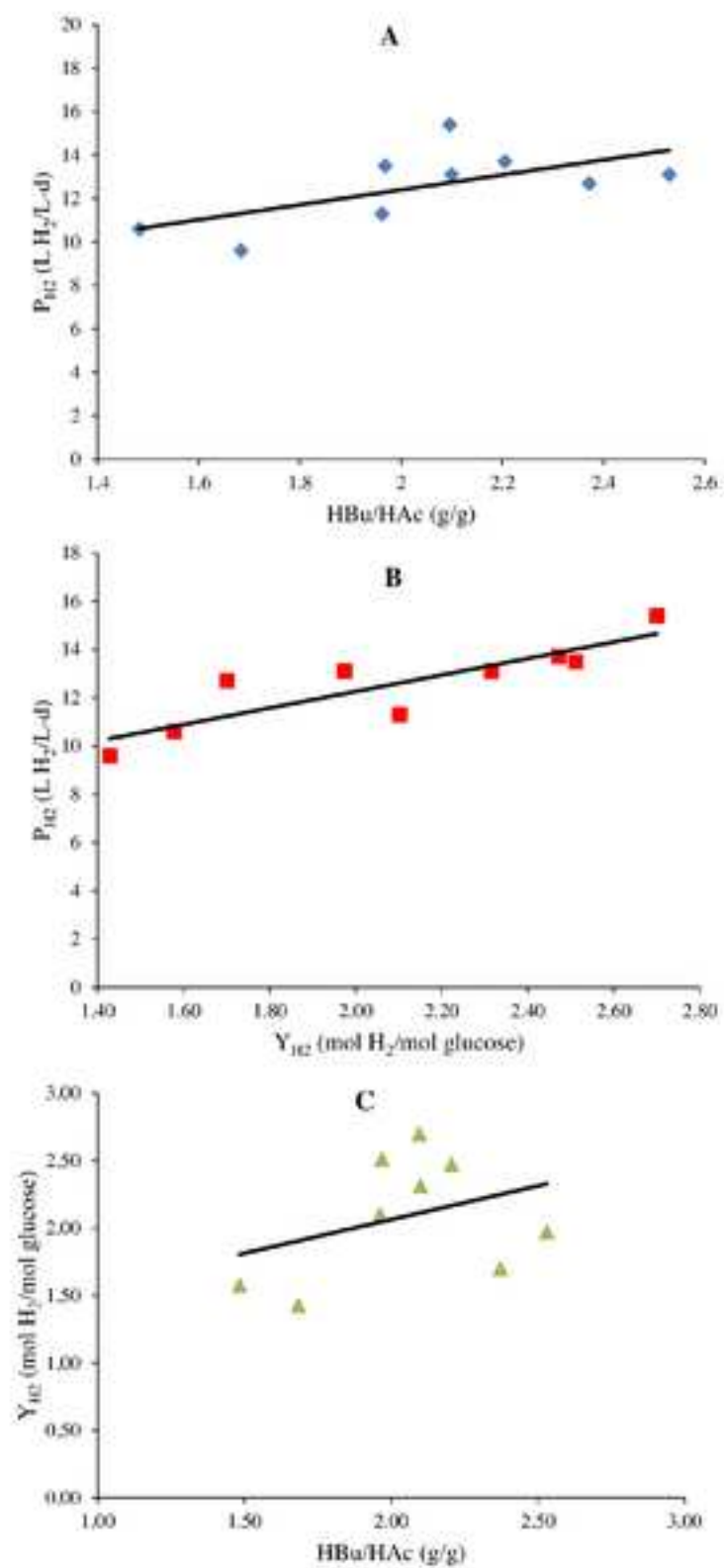




Table 1

Reactor 1				Reactor 2				
HRT (h)	Duration (h)	P <sub>H2</sub> (L H <sub>2</sub> /L-d)	Y <sub>H2</sub> (mol H <sub>2</sub> /mol glucose)	HRT (h)	Duration (h)	HBu/HAc (g/g)	P <sub>H2</sub> (L H <sub>2</sub> /L-d)	Y <sub>H2</sub> (mol H <sub>2</sub> /mol glucose)
6	24	18.1	1.82	10	25	1.48	10.6	1.58
6	24	18.1	1.94	10	18.3	1.68	9.6	1.43
6	19.6	17.7	1.88	10	11.4		9	1.34
5	6	17.3	1.29	5	6		12.7	1.06
6	26.2	18.5	1.86	10	23	1.96	11.3	2.10
6	24	16.3	1.64	10	27.5		9.8	1.82
6	24	17.3	1.78	10	25		11.2	2.04
5	24	17.1	1.45	10	20.6	2.10	13.1	2.31
6	32.7	16.7	1.58	5	24		17.1	1.59
6	24	15.8	1.59	10	25	1.97	13.5	2.51
6	26.2	16.2	1.63	10	25	2.20	13.7	2.47
3	6	15.6	0.80	10	27.5	2.53	13.1	1.97
6	30.5	17.1	1.37	3	6		14.6	0.81
6	13	17.9	1.92	10	27.5		13.8	2.57
3	24	19	0.95	10	23	2.37	12.7	1.70
6	32.7	18.1	1.88	10	23		11	2.05
6	24	16.7	1.29	3	24		18.5	0.98
6	19.6	17.1	1.60	10	29.7	2.10	15.4	2.70
6	17.5	17.3	1.68	10	23		13.8	2.49
				10	25		15	2.37

ANOVA RESULTS (based on P<sub>H2</sub>)

Variables (Reactor 1)		Variables (Reactor 2)	
	p-value		p-value
HRT	0.967	HRT	<b>0.0008</b>
Duration	0.327	Duration	<b>0.0012</b>
HRT:duration	<b>0.016</b>	HRT:duration	0.7798
Bartlett test	0.072	Bartlett test	0.7606

p<0.05 is considered statistically significant